Sveriges lantbruksuniversitet
Fakulteten för veterinärmedicin och husdjursvetenskap

Swedish University of Agricultural Sciences
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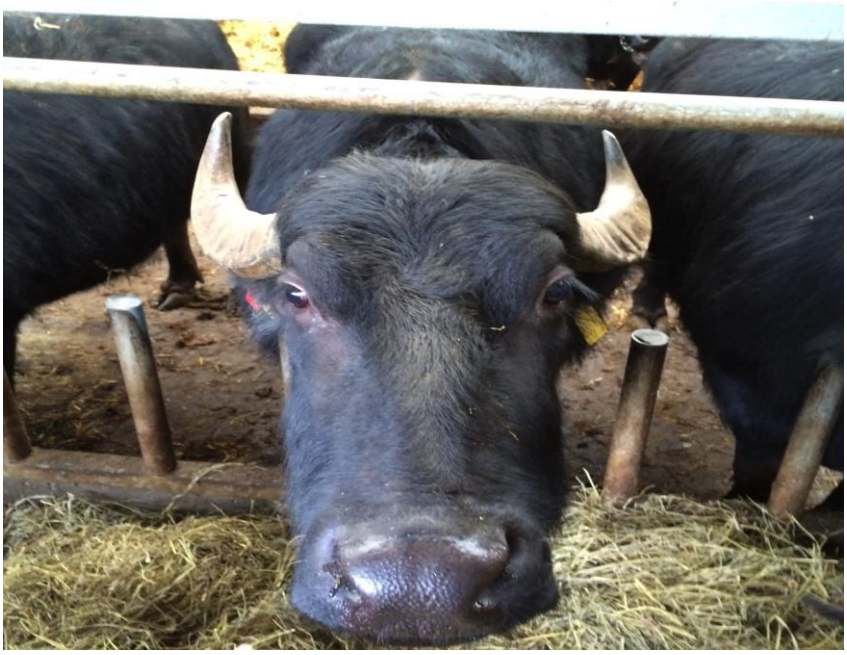
Composition of water buffalo milk during the first period of lactation

- Relation to mozzarella cheese properties



Mokhtar Kashwa

Examensarbete / SLU, Institutionen för husdjurens utfodring och vård, **548**

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Sammansättning av vattenbuffelmjölk efter kalvning i förhållande till mozzarella egenskaper

Mokhtar Kashwa

Handledare:
Supervisor: Monika Johansson, SLU, Department of Food Science
Examinator:
Examiner: Kerstin Svennersten Sjaunja, SLU, Department of Animal Nutrition and Management
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Abbreviations

AAS	Atomic absorption spectrophotometry
CCP	Colloidal calcium phosphate
CE	Capillary electrophoresis
CMP	Casein macro peptide
DTT	DL-dithiothreitol
FFA	Free fatty acids
GMP	Gluco macropeptide
GT	Galactosyltransferase
IDF	International Dairy Federation
MHEC	Methylhydroxyethylcellulose
MOPS	(N-morpholino) propanesulfonic acid
NPN	Non protein nitrogen
PDO	Protected Designation of Origin
SCC	Somatic cell count
TN	Total nitrogen
Tris	(Tris) Hydroxymethyl-aminomethane
TS	Total solids
TSG	Traditional Specialty Guaranteed
α-LA	α-lactalbumin
αs1-CN	αs1-casein
αs2-CN	αs2-casein
β-CN	β-casein
β-LG	β-lactoglobulin
κ-CN	κ-casein

Content

Abstract

Fresh buffalo milk is the raw material used traditionally to prepare mozzarella cheese by hand. The characteristic smooth texture of mozzarella is obtained through a unique step during its manufacturing process, which is plasticizing or stretching of the cheese curd in hot water. Buffalo milk during about one month after calving is unsuitable for mozzarella production as reported by Ängsholm dairy farm in Uppsala, Sweden, and also by other mozzarella producers.

The aim of this study was to investigate changes in milk composition in lactating water buffaloes for about five weeks after calving in relation to mozzarella cheese properties. Individual and bulk milk samples from six Mediterranean buffaloes were analyzed in terms of milk composition, total calcium content, protein profile and ionic calcium concentration. Milk gross composition was analyzed based on Fourier Transform Infrared Spectroscopy. Total calcium content was analyzed by atomic absorption spectrophotometry according to IDF 119: 2007 and IDF 154: 1992. Milk protein analysis was done by capillary electrophoresis. Ionic calcium was determined using calcium ion-selective electrode.

Results showed that total calcium content of buffalo milk during the first five weeks of lactation was lower than reported for the average buffalo milk in mid and late lactation. The result of calcium activity of the milk was found to be in favor of obtaining a harder gel. Milk protein analysis revealed that buffalo milk in the first period of lactation is considerably lower in κ-CN and β-CN concentrations than the reported concentration in buffalo milk.

Sammanfattning

Färsk buffelmjölk är råvara till traditionell hantverksmässig mozzarellaframställning. Den karaktäristiskt smidiga mozzarellakonsistensen erhålls genom ett unikt steg i tillverkningsprocessen, sträckning av ostmassa i varmt vatten. Under cirka en månad efter kalvning är buffelmjölk olämpligt för mozzarellaframställning, vilket meddelats av Ängsholmens gårdsmejeri utanför Uppsala men också av andra mozzarella producenter.

Syftet med denna studie var att undersöka förändringar i mjölkens sammansättning hos vattenbufflar i cirka fem veckor efter kalvningen i relation till mozzarellaostens egenskaper. Individuella och tankmjölksprover från sex medelhavsbufflar analyserades med avseende på mjölkens sammansättning, den sammanlagda kalciumhalten, protein-sammansättningen och koncentration av joniskt calcium. Mjölkens bruttosammansättning analyserades baserad på FTIR (Fouriers Transform Infrared Spectroscopy). Sammanlagt calciuminnehåll analyserades med atomabsorbtionsspektrofotometri enligt IDF 119: 2007 och IDF 154: 1992. Analysen av mjölkprotein gjordes med kapilärelektrofores. Joniskt calcium bestämdes genom kalciumjonselektiv elektrod.

Resultaten visade att den sammanlagda kalciumförekomsten i buffelmjölk under de fem första laktationsveckorna var lägre än vad som tidigare redovisats. Resultatet av mjölkens kalciumaktivitet var fördelaktigt för att erhålla en hårdare gel. Slutligen visade analys av mjölkprotein att buffelmjölk i början av laktationen har lägre värde när det gäller koncentration av κ-CN och β-CN än vad tidigare redovisats.

1. Introduction

1.1 Background

Mozzarella is a member of the *pasta filata* or stretched curd cheese family that originated in Italy and was originally made from high fat buffalo milk. Manufacturing of mozzarella by hand can be challenging and is a real work of art and requires a lot of experience. Mozzarella gets its characteristic smooth texture through a unique step during its manufacturing process, which is plasticizing and texturing treatment of the fresh curd in hot water, which gives the finished mozzarella its fibrous structure and melting characteristics. The finished mozzarella is white, soft, unripened and is intended to be consumed shortly after manufacture. Mozzarella is one of the main ingredients in the Italian cuisine and is a key part in the manufacture of Pizza. Mozzarella is famous and consumed worldwide although it originated from Italy. It can be made from buffalo milk, cow milk or from a mixture of both.

The European Union granted Italy an official registration of protected designation of origin (PDO) for their mozzarella production in 1996 and since then the Italian farmers and mozzarella producers in the Campana region are able to label their mozzarella production with PDO. Italy was also granted the traditional specialty guaranteed (TSG) for mozzarella the same year, which enables the mozzarella producers within the EU to label their mozzarella production with TSG provided that they follow the recipe of mozzarella production with buffalo milk, cow milk or a mixture of them. There are differences between the PDO and TSG. The PDO is stricter and offers a governmental protection for the original product to keep up with competition in the market against the cheaper products of inferior quality. Protection provided by the PDO enables the small-scale producers to make benefits and to go forward with their mozzarella production. The PDO protects also the consumers ensuring that they get a registered product with high quality.

Milk composition changes through seasons and stages of lactation (Tsioulpas *et al.*, 2007a). Ängsholm dairy farm, Uppsala, Sweden is a Mediterranean buffalo dairy farm where buffalo milk is used to produce mozzarella cheese by hand. A frequent observation was reported that the buffalo milk during approximately the first five weeks after calving is not suitable for mozzarella manufacturing. This is due to the failure of the curd to be stretched even with modification of the milk (pH adjustment or $CaCl_2$ addition). Studies on buffalo milk are limited compared to studies carried out on cow milk and there is a need to fill up the gap with new knowledge.

1.2 Hypothesis and objectives

The first buffalo milk cannot be used in mozzarella manufacturing as reported by the Ängsholm dairy farm. It takes about one month after calving until the milk becomes suitable for mozzarella production. No studies could be found on this topic.

The hypothesis was that the composition of milk during the first period after calving in buffaloes is different than buffalo milk in mid or late lactation, which might affect manufacturing properties of mozzarella. Thus, the overall objective of this study was to

investigate changes in milk composition in six lactating water buffaloes for about five weeks after calving in relation to mozzarella cheese manufacturing properties.

Specific goals of the study were to:

1. Determine the gross composition (fat, total protein, lactose, total solids etc.) of both bulk tank milk and milk from six individual water buffaloes.
2. Measure the pH and free Ca^{2+} ion concentration of milk.
3. Determine the total calcium content in milk.
4. Analyze the milk protein profile.

2. Literature review

2.1 Water buffalo (*Bubalus bubalis)*

Buffalo is an economically important multipurpose livestock animal especially in the low-income countries and plays a significant role through the contribution in many cultural and social aspects. Domestic water buffalo (*Bubalus bubalis)* is a member of the family *Bovidae*, which can be classified into two main classes: river buffalo and swamp buffalo. Swamp buffalo has low milk production (1.0-1.5 liter per day) compared to water buffalo. Due to their low productivity of milk, they are mainly used as draught animals in rice fields in China, Indonesia, Philippines and other Southeast Asian countries. The river buffalo, including about 18 breeds (e.g. Murrah, Nili-Ravi, Mediterranean and Jaffrabadi), are mainly kept for milk production with a daily average from 6-7 liters of milk. They originated from India where their natural habitat is close to rivers due to their preference to wallow in running water (Thomas, 2008).

Dairy buffalo production has been a tradition in many parts of the world like Asia (particularly India, Pakistan and China), Egypt, Caucasian and Balkan countries. Buffalo milk and dairy products have recently become very popular and sought after, especially mozzarella cheese made from buffalo milk, which led to increase in wide distribution of buffalo even in non-traditional areas. Italy has a flourishing dairy buffalo industry and is famous worldwide for its cuisine in which buffalo mozzarella cheese is one of the major and popular ingredients. Numbers of buffalo animals are present nowadays across Europe (Germany, Sweden, UK) to South America (Brazil, Argentina) and the USA (Thomas, 2008).

The buffalo population is about 170 million heads worldwide with the majority found in Asia (97%) and 2% in Africa (of which 98% in Egypt). The buffalo population in India represents about 56%, Pakistan 14% and China 13% of the total buffalo population in the world. Buffaloes are the second largest source of milk supply in the world with the total global milk production estimated to be about 72 million tons. Despite the important contribution of buffalo in dairy and milk production especially in the developing countries, very little resource and international effort as well as few studies have focused in developing this species compared to cattle. The genetic potential of the buffalo is still not fully used and most of the buffalo production is done by small-scale holders (Thomas, 2008).

2.1.1 Lactation in buffalo

The lactation period starts with the birth of the calf and the initial milk yield is a reliable indicator of the genetic potential of the animal. The udder secrets colostrum during about six days from calving date. Colostrum is used for the feeding of the calf owing to its defensive and nutritious properties played by the high content of vitamins and immunoglobulins. Colostrum is different from normal milk secreted throughout the lactation period and it cannot be used for dairy production. The highest milk yield is reached after five to six weeks after calving. When buffalo cows stop producing milk, the lactation ends and they enter the dry period. The optimal lactation length in Murrah buffalo has been reported to be 260 to 295 days whereas in Italy, the Mediterranean buffalo has a lactation length of 270 days (Catillo *et al.*, 2002; Thomas, 2008).

2.1.2 Factors affecting lactation and milk yield

Lactation and milk yield depend on many factors including genetic and non-genetic aspects. The genetic factors are the breed and individual genetic make up. The non-genetic factors include feed quantity and quality, animal health status, farm management and environmental factors. The shape of lactation curve depends on many factors including nutrition, milking frequency, management and health condition. The most important factor for increasing and sustainability of the milk yield is feeding. The dry period can also have an effect on the next lactation and milk yield as the milk yield in the subsequent lactation increases with increasing length of the dry period (Sørensen and Enevoldsen, 1991). Buffalo cows should be dried off about two to three months before next calving. The dry period allows the buffalo to rest and to reconstruct the udder tissue (Catillo *et al.,* 2002; Thomas, 2008).

2.2 Difference in composition between buffalo and cow milk

Differences in composition between buffalo milk and cow milk have been reported by many workers (Ahmad *et al.,* 2008; Zicarelli, 2004; Spanghero and Susmel, 1996). Fat content was found to be higher in buffalo milk, compared to cow milk in many studies. The average fat content in buffalo milk is about 7 to 8% (Thomas, 2008). A study by Varrichio *et al.,* (2007) on milk fat of Mediterranean buffalo milk showed that the fat content in buffalo milk averages 8.3% and can reach up to 15% under normal conditions. Fat globules in buffalo milk are bigger than in bovine milk with 60% having a size between 3.5 to 7.5 µm (Ahmad *et al.,* 2008). El-Zeini (2006) even reported a much larger diameter (8.7µm) than cow fat globules (3.95 µm).

Buffalo milk fat has a higher melting point than cow milk as it contains higher amounts of saturated fatty acids and lower amounts of unsaturated fatty acids than cow milk. (Varrichio *et al.,* 2007; Thomas, 2008). Buffalo milk has also higher contents of palmitic, butyric, and stearic acids and lower amounts of caproic, caprylic and capric acids. Cow milk is more susceptible to oxidative changes than buffalo milk (Thomas, 2008). Zicarelli (2004) reported that milk and mozzarella phospholipids and cholesterol content is lower for buffalo than cow milk even if buffalo milk has higher fat percentage. Buffalo milk contained 275 mg cholesterol whereas cow milk contained 330 mg. Buffalo milk has higher dry matter content, which plays a role in the higher cheese yield, compared to bovine milk (Zicarelli, 2004).

Moreover, buffalo milk has higher concentrations of protein, ash and lactose than cow milk (Ahmad *et al.,* 2008). Protein content in buffalo milk ranges from 4.2 to 4.5% while cow milk has 3.6% protein (Thomas, 2008). Casein concentration is higher in buffalo milk than cow milk with a higher casein index (casein content/protein content x 100). The casein index of buffalo milk is higher than 80%. Spanghero and Susmel (1996) reported that the concentration of protein is 30%, calcium 70% and phosphorus 30% higher in buffalo milk than cow milk. Buffering capacity of buffalo milk is higher than cow milk. Casein micelles in buffalo milk has less hydration and more mineralization than cow milk which means that the buffalo milk contains more minerals as calcium, magnesium etc. (Ahmad *et al.,* 2008).

The content of lactose was reported to be slightly higher in buffalo milk (about 5%) than in cattle milk (4.8%). Buffalo milk is higher than cow milk in Total solids (TS) as buffalo milk has 16% TS while cow milk has 13% TS (Thomas, 2008). The pH of buffalo milk is 6.81 while pH of the cow milk is 6.76 (Ahmad *et al.,* 2008).

The composition of the milk changes during lactation period and through season. Tsioulpas *et al.,* (2007a) analyzed samples from individual cows from the colostrum, postcolostrum and early lactation to investigate changes in milk composition and its suitability for processing. They found that the pH was very low in the beginning and showed a steady increase thereafter. Fat content varied throughout the sampling period but it showed neither increased nor decreased trend. Total protein content declined steadily until 30 days after calving in contrast to lactose content which increased continuously during the same period.

The normal somatic cell count (SCC) in milk from buffalo cows is less than $5*10^5$ cells/ml (Priyadarshini and Kansal, 2002). Singh and Ludri (2000) reported that the lowest value of SCC in buffalo milk is reached during 90 to 150 days of lactation. No differences in SCC between morning and evening lactations were found. Stage of lactation had no effect on the milk SCC whereas season had a significant effect. A significant negative correlation was found between milk yield and SCC during different stages of lactation (Singh and Ludri, 2000). Priyadarshini and Kansal (2002) investigated the lysozyme activity in buffalo milk and reported that some buffalo cows showed 1000 fold higher lysozyme activity and moderately raised SCC in milk without showing signs of mastitis.

2.3 Mozzarella cheese

2.3.1 Manufacturing of mozzarella

Manufacturing of mozzarella cheese starts with standardization of the milk as the milk fat % is adjusted to 3.0-6.0% (Figure 1) to obtain mozzarella cheese of satisfactory quality. Variations in standardization exist depending on the manufacturing process and type of mozzarella. The optimal fat content for mozzarella used in pizza topping is 2.5%. Heat treatment of milk is done at 71.7 °C for 15 seconds because the temperature at the plasticizing step is not enough to kill pathogens in milk. Pasteurization of milk enhances flavor and keeping quality of mozzarella. Cow milk is homogenized to obtain a whiter cheese with enhanced flavor. Homogenization affects properties of the resultant cheese as curd stretching, fat leakage at baking, cheese moisture and yield. Various levels of homogenization pressure are applied in different recipes. This step is skipped in the TSG recipe. The following step is inoculation of the starter culture. *Streptococcus thermophilus* is the only starter used for fresh Italian mozzarella whereas the moisture content of mozzarella determines the kind of starters used. *Streptococcus thermophilus*, *Lactobacillus helveticus* and *Lactobacillus bulgaricus* are used to produce mozzarella with low moisture content, while *Streptococcus lactis* and *Streptococcus durans* can be used to produce high-moisture mozzarella (Jana and Mandal, 2011; Calandrelli, 1997).

Milk is then inoculated with animal rennet, which is extracted from the abomasum of young ruminants as calves, lambs and kids. Bovine rennet is the type used in the TSG recipe. Liquid animal rennet is added to cheese milk initiating milk coagulation at the temperature of 36-38 ° C. Setting of the curd starts with renneting forming the first floccules of the gel. The gel undergoes hardening and consequently becomes compact and homogeneous. The curd is cut into large cubes and separation between curd and whey occurs. The large cubes are thereafter cut into walnut size granules, which are soft and rich in whey. The curd is then gently stirred. This step is important for the final yield and composition of mozzarella. Up to 1% of fat can be lost during this step and therefore, an efficient cutting of the curd is crucial to decrease fat losses as possible. Maturation of the curd occurs to a pH of 5.4-5-0 and takes place under whey. The curd is stretched at the end of maturation stage at the optimal pH of 4.9 in hot

water. The hot water stretching step (also known as plasticizing) is characteristic of mozzarella manufacture. The temperature of water used in stretching step is 95 °C to reach a final curd temperature of 65-85 °C. Manual stretching of the curd is a difficult process and requires experience. Stretching ends when the cheese becomes homogeneous and shiny and ready for shaping. In semi-industrial dairies, shaping is totally mechanized whereas in farm dairies the cheese curd is shaped manually and cut into spherical shapes of 20-220 g. The resulting mozzarella is kept in cold water and packed. Salt can be added if desired. Mozzarella is finally stored at +4 °C in brine (Jana and Mandal, 2011; Calandrelli, 1997).

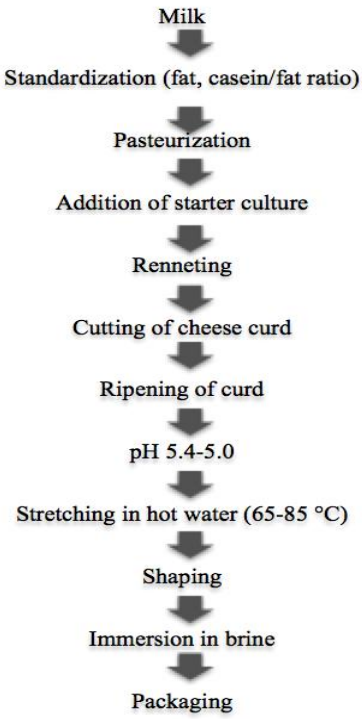

Figure 1 Flow chart of mozzarella cheese manufacturing (Jana and Mandal, 2011).

2.3.2 Composition of mozzarella cheese

The composition of mozzarella cheese is dependent on type of milk (cow vs. buffalo milk or a mixture of them). It depends also on the manufacture procedure followed by the producer. Table 1 shows composition of mozzarella cheese. Cholesterol concentration in the finished mozzarella cheese is also lower when using buffalo milk compared to cow milk; the buffalo mozzarella contains 1562 mg kg^{-1} whereas cow mozzarella contains 2287 mg kg^{-1} (Zicarelli, 2004).

Table 1 Composition of mozzarella cheese (adapted from Jana and Mandal, 2011)

Constitutes (%)	Buffalo milk[1]	Cow milk[2]	Cow milk[3]
Moisture	50.51	49.30	48.59
Fat	26.34	25.85	27.25
Protein	20.48	20.83	20.06
Lactose	-	-	1.59
Ash	2.0	3.62	2.25
Salt (NaCl)	0.90	2.17	-
Acidity	0.61	0.76	0.66

[1] Jana and Upadhyay (1992), [2] Pizaia *et al.,* (2003), [3] El-Owni and Sana (2009)

2.4 Calcium in buffalo milk

2.4.1 Calcium content in buffalo milk

Calcium content in buffalo milk varies according to time of the year and stage of lactation. It was reported by Patino *et al.,* (2007) that the mineral content (Ca, P, Na, Cu and Fe) in buffalo milk varies from a period to another throughout the year. Patino *et al.,* (2007) reported also that winter is the time of the year when milk mineral content becomes the lowest. Mineral content can also be influenced by stage of lactation of the buffalo. The last third of lactation was shown to be the period with the lowest mineral content (Ca, P, K, and Cu) (Patino *et al.,* 2007).

Buffalo calcium content was measured and reported in different studies. Ariota *et al.,* (2007) measured calcium content in 70 different Italian buffaloes from three farms in mid lactation. Ariota *et al.,* (2007) reported that calcium content was 1.7 g kg^{-1}, which is less than 2.03 g kg^{-1}, reported by Spanghero and Susmel (1996), who measured calcium content in 25 buffaloes in different stages of lactation. Elvingson (2014) measured calcium content of milk (individual and bulk) obtained from seven Mediterranean buffaloes from Ängsholm dairy, Uppsala, Sweden. The buffaloes were in late lactation when the individual and bulk samples were collected. Bulk milk calcium content was about 2.3 g kg^{-1} while mean individual milk calcium content was 1.72 g kg^{-1} (Elvingson, 2014).

2.4.2 Calcium content in the milk of other animals

Bovine milk is characterized by the super saturation of calcium phosphate and thus it is considered to be an excellent source of calcium for the newborn. Fox and McSweeney (1998) reported that 500 mg of calcium are there per one litter of cow milk in colloidal form. In milk from cows and goats, about 65% of calcium is associated with casein unlike the human milk, which has a low level of casein with about 25% of calcium associated with casein. Rat milk is very rich in casein content with about 95% of calcium associated with casein (Neville *et al.,* 1994). Tsioulpas *et al.,* (2007a) reported that the total calcium content in cow milk showed a steady decline during the first 30 days after calving, measuring 1.44 g kg-1 on day five, 1.34 g kg-1 on day 15 and 1.21 g kg-1 on day 30.

2.4.3 Free Ca^{2+} ion activity and concentration in milk

In bovine milk, calcium content is mainly distributed between the casein micelles in the colloidal phase and the aqueous phase. Calcium in the aqueous phase is distributed as ionic calcium (Ca^{2+}), calcium citrate and calcium phosphate. It seems that the calcium distribution between the two phases (aqueous and colloidal) depends on the casein level in milk (Holt and Jenness, 1984; Neville *et al.,* 1994). Calcium activity in milk is the uncorrected direct measurement of Ca^{2+} concentration and information on its properties in the aqueous phase is still limited (Tanaka *et al.,* 2011).

Ca^{2+} concentration can be measured by different methods. It can directly be determined using calcium ion-selective electrode (Allen and Neville, 1983). It can be measured using ultrafiltration and equilibration dialysis methods (Silanikove *et al.,* 2003). No studies could be found about measuring Ca^{2+} concentration in buffalo milk.

Silanikove *et al.*, (2003) analyzed the calcium ion concentration in milk from different animal species (including cow, goat, sheep) using a calcium ion-selective electrode. They reported that Ca^{2+} concentration in all these species was between 3.2-4.2 mM while there was a distinct difference in total concentration of calcium from one species to another. The study suggested also that casein (being a chelating agent) interferes to a large extent with measurement of the calcium ion. The interfering effect of casein on measurement increases with the increase in casein concentration. Direct measurement of calcium ion activity using an ion electrode showed lower values than when using the other measurement methods i.e. equilibration dialysis or ultrafiltration.

Measurement of calcium ion activity is reported to be affected by some important factors e.g. the type of method used for measurement and temperature (ambient, electrode and milk sample). Landenson and Bowers (1973) found that the value of calcium ion concentration recorded at 25 °C to be higher by 2% to 5% than that recorded at 37 °C. Changes in pH were found to affect the measurement of calcium activity when measured using an ion-selective electrode (Allen and Neville, 1983).

Tanaka *et al.*, (2011) measured calcium activity in bovine milk using an ion-selective electrode in relation to the hot season. The average of calcium activity was found to be 1.26 mM in the period from June to October with a marked decrease in the period from July to August. The total calcium content was also measured and the result was 1.05 g kg^{-1} for the entire period with a marked decrease in concentration during July. They reported also that the ratio between total calcium content to calcium activity was 4.92% throughout the experiment and decreased markedly during August. The calcium activity in milk was found to be from 4% to 6% of the total calcium content. No correlation was found between total calcium content and calcium activity whereas a positive correlation between calcium activity and milk yield was found. A negative correlation between calcium activity and milk lactose was also observed. Holt *et al.*, (1981) reported that calcium ion concentration in bovine milk was 2.0 mM and that the ratio between total calcium content to calcium activity was about 6.9%.

2.5 Calcium role in manufacturing of mozzarella cheese

Mozzarella cheese is famous for its unique fibrous structure that results from a characteristic last step in the manufacturing process known as the plasticizing step. At this stage, stretching and kneading of the cheese curd occur in hot water (65-85 °C). During stretching of the curd, rearrangement of protein fibers is achieved in the same direction of stretching. A low calcium and PO_4 content in the curd together with a high concentration of intact casein is crucial to obtain appropriate fibrous threads during stretching under hot water (Lucey and Fox, 1993).

2.5.1 Reduction of pH

Obtaining optimal pH before renneting helps to get a gel of suitable consistency. When starting the coagulation process with a high pH it leads to a low whey calcium concentration than when starting with a low pH (Yazici and Akubulut, 2007). Fox and McSweeney (1998) reported that clotting time increases when pH increases and decreases when protein content increases. The reduction of pH can be done with two methods; the first method is via addition of a weak acid or a mixture of acids (direct acidification) to obtain a specific pH at which stretchability of the curd is optimal. Examples of the acids that can be added are lactic and citric acids. The second method to reduce pH is a microbiological method by adding the

starter culture, which breaks down lactose into lactate. The pH reduction occurs usually after milk pasteurization and directly before renneting. It is an important step because pH reduction helps to activate rennet, which lead to a faster formation of the curd. The reduced pH leads also to increased aggregation of para-casein micelles via reduction of the net charge of the casein micelles (Guinee *et al.,* 2002; Kindstedt, 2004).

2.5.2 Coagulation of the curd

This step starts with the addition of rennet to the milk without stirring. Many enzymatic activities occur during this process, as the rennet initiates the breakdown of κ-CN, which enables the activity of hydrophilic caseinmacropeptide (CMP) to diffuse in the solution. This leads to aggregation of the casein micelles followed by formation of gel network through syneresis of water and whey proteins out of the curd.

There is an inverse relationship between Ca^{2+} level and coagulation time. It was also found that if the Ca^{2+} level is lower than 1.5 mM, no coagulation will occur, which suggests that a minimum Ca^{2+} concentration is necessary for coagulation to get started (Tsioulpas *et al.,* 2007b). Lucey and Fox (1993) indicated that it was not possible to restore curd firmness of cow milk with poor renneting properties in late lactation neither by pH adjustment nor addition of calcium.

2.5.3 Plasticizing and stretching of the cheese curd

The curd stretchability depends on pH and curd demineralization. The optimal pH for stretchability is 5.0-5.2. Aggregations of small caseins occur at this pH with the increased application of heat forming long chain structures. Lucey and Fox (1993) reported that the crosslinks between protein fibers could be reduced with the decreased calcium levels. When pH is less than 4.8, it does not allow crosslinks between protein fibers and leads to loss of curd stretchability (Lucey and Fox, 1993). Yazici and Akubulut (2007) reported that milk with lower pH at coagulation of the curd takes less time to reach the optimal pH (5.0-5.2) and vice versa. Plasticizing of the curd depends on curd pH, which in turn depends on calcium content. When calcium is reduced, the crosslinking will be affected resulting in higher flowing properties of the curd (Metzger *et al.,* 2001).

2.5.4 Preacidification of milk before plasticizing

Preacidification is the step that takes place before the coagulation (setting time) to lower the micellar calcium concentration by adding weak acids before starter culture addition. Low calcium concentration at setting time is shown to be preferable to obtain ideal curd stretchability during the plasticizing stage. Metzger *et al.,* (2000) reported that addition of weak acid to milk before the addition of rennet at the time of coagulation reduces calcium concentration in the finished mozzarella. By this way, the pH is lowered resulting in reduction of coagulation time from 15 to 30 minutes. They reported also that calcium concentration increased in whey whereas calcium concentration was reduced in the final cheese. Metzger *et al.,* (2000) defined the factors on which reduction of curd calcium concentration depends. These factors are type of acid used, reduction of pH at setting time and whey drainage. As well, Joshi *et al.,* (2003) reported that addition of weak acid to milk to decrease the micellar calcium affects the rheological properties of the finished cheese. It also shortened the time needed to melt and soften the cheese.

2.6 Milk proteins

The total protein component of milk is composed of a heterogeneous group of numerous proteins, which plays vital biological functions (Ng-Kwai-Hang, 2002). The primary group and the most important proteins in milk are caseins and whey proteins. About 80% of total bovine milk protein is in the form of casein, whereas the other 20% of milk protein consists of whey (serum) protein (Walstra *et al.*, 2006). Ahmad *et al.*, (2008) analyzed the overall composition of buffalo and cow milk. Buffalo milk contained total nitrogen (TN) of 43.5 g kg^{-1}, which is higher than TN in cow milk (33.5 g kg^{-1}). Bonizzi *et al.*, (2009) reported similar results that TN in buffalo milk (43.4 g kg^{-1}) was higher than TN in cow milk (33.5 g kg^{-1}). Such as in cow milk; caseins in buffalo milk accounts for about 80% of total protein and the rest 20% whey protein (Ahmad *et al.*, 2008). Studies have been carried out on buffalo milk proteins but not as extensive as those concerning cow milk proteins.

2.6.1 Casein micelles

Casein is found in milk in the form of micelles with the presence of calcium phosphate (Ng-Kwai-Hang, 2011). Casein micelles are a colloidal suspension of calcium phosphate in milk and their size in bovine milk ranges from 50 to 500 nm. Their loose structure can be disrupted by addition of acid or alkali to milk as well as when exposed to heat (Ng-Kwai-Hang, 2002). Increase of any negative charge (citrates or phosphates) increases casein micelle stability in milk while increasing amount of calcium ions destabilizes the micelles. The micellar structure is still open to debate and much controversy exists over the existence of secondary structure in the caseins. Different theories suggest the possible micellar structure and function. Holt (1995) suggested that the primary function of caseins is not nutritional rather than preventing pathological calcification of the mammary gland through transportation of calcium and phosphate via casein micelles. However, the function is also to provide calcium to the offspring in the first stage of life (Holt, 1995).

Differences between buffalo and cow casein micelles were reported. Ahmad *et al.,* (2008) indicated that the water content of ultracentrifuged casein pellets was higher in cow milk (2.24 g water/g of dry pellet) than buffalo milk (1.90 g water/g of dry pellet) at normal pH. Ahmad *et al.,* (2008) reported also that buffalo milk has higher casein content (34.6 g kg^{-1} for buffalo milk and 26.1 g kg^{-1} for cow milk) and higher mineralization of casein micelles than cow milk. Bonizzi *et al.*, (2009) reported similar results regarding casein content; 34.6 g kg^{-1} for buffalo milk and 26.1 g kg^{-1} for cow milk. Sabarwal and Ganguli (1977) reported that buffalo milk had κ-CN with less sialic acid than κ-CN in cow milk and that the glycopeptide released from κ-CN in buffalo milk was lower in molecular weight than that in cow milk. McMahon and Oommen (2008) presumed an interlocking lattice model of the casein micelle based on high-resolution transmission electron microscopy (TEM) micrographs of freeze dried immobilized casein micelles. Calcium phosphate nanoclusters are presumed to be present at the interlocking sites and to maintain supramolecule integrity and an integral role in casein micelle integrity. Aggregates of calcium phosphate-casein act as structure-forming sites, which bind other caseins forming short chains (McMahon and Oommen, 2008).

Holt (1995) suggested a model called calcium phosphate nanoclusters, which is small aggregations of micellar calcium phosphate (MCP) involving the serine phosphate groups of the caseins and possible also glutaminate residues, forming centers from which casein micelles grow. This model cannot simply explain the reason behind the almost continuous outer hairy layer of κ-CN of the casein micelles with little κ-CN in the micellar core.

The classical model of bovine casein structure has emerged based on continuous observations by many workers. Some important features of this model include: casein micelles do not possess smooth surface, yet they are roughly spherical in shape, sub-micelles have mixed composition and the sub-micelles are of two types; one containing mainly αS2-CN and β-CN whereas the other containing αS1-CN and κ-CN, sub-micelles can be linked together through calcium phosphate bridges with the κ-CN to the outside (Walstra, 1990). The controversy continues to the present moment with many evidences for and against the sub-micelles and many modifications have been proposed by many workers.

The casein micelles are very stable. Steric repulsion caused by κ-CN hairs leads to caseins stability against aggregation. β-CN is also believed to help in stability of caseins at low temperature. Aggregation or disintegration may occur due to changes in conditions. Gel is formed due to aggregation of caseins. Colloidal calcium phosphate (CCP) binds the submicelles together with controversy still ongoing about the actual structure and composition of CCP (Walstra, 1990).

Lowering pH leads to considerable changes in the casein micelles. The CCP moves into the solution leaving micelle-like particles, which have different properties than the casein micelles. Slight changes in voluminosity and size distribution occur within the pH range of 6 to 6.6 but needs further investigation. Near the pH of 5.2, critical transitions occur which affects the elastic properties of the renneted milk. Near that pH, bonds that keep the casein micelles together are weakest leading to the optimal meltability or stretchability of the curd (Walstra, 1990).

2.6.2 Caseins

Caseins are a group of proteins specific for milk forming about 80% of total bovine and buffalo milk protein. Caseins are classified according to their net charge into four distinct molecules: αS1-casein (αS1-CN), αS2-casein (αS2-CN), κ-casein (κ-CN) and β-casein (β-CN). Caseins are negatively charged hydrophobic molecules possessing a high content of the amino acid proline (Ng-Kwai-Hang, 2002; Ng-Kwai-Hang, 2011). The αs2-CN possesses ten proline amino acids and two cysteine residues linked by a disulfide bond. αs2-CN is the most hydrophilic of all the caseins and has the ability to form dimers and appears as multiple peaks in capillary electrophoresis analysis. αS1-CN is formed of 199 amino acid residues with three hydrophobic regions: one region at each end and the polar region where most of the proline is located. αS1-CN is the highest charged of all caseins and is less sensitive to calcium than αS2-CN.

In contrast to αs2-CN, β-CN is the most hydrophobic of all the caseins. β-CN has no cysteine and possesses numerous proline residues located at its hydrophobic part. The N-terminal of the β-CN polypeptide chain has a strong negative charge whereas the hydrophobic part lacks net charge. Due to its amphiphilic structure (i.e. contains both hydrophobic and hydrophilic groups), β-CN gets its arrangement of micelles' aggregates in the milk (Ng-Kwai-Hang, 2002; Walstra *et al.*, 2006, Ng-Kwai-Hang, 2011). Increased β-CN concentration has been correlated with enhanced cheese-making properties as increasing whey expulsion and reducing rennet-clotting time (Wedholm *et al.*, 2006, Jimenez and Richardson, 1988). κ-CN plays an important role in casein micelle stability against precipitation by calcium ions due to its poor calcium binding ability (Ng-Kwai-Hang, 2011). κ-CN is water soluble due to its hydrophilic C-terminal. κ-CN properties are used in cheese manufacture when renneting the milk. κ-CN is linked to improved heat stability and enhanced cheese-making properties

(Wedholm *et al.*, 2006, Jimenez and Richardson, 1988). Rennet cuts its C-terminal and as a result, a free glucomacropeptide (GMP) or caseinmacropeptide (CMP) and hydrophobic aggregation of the casein micelles are gained (Walstra *et al.*, 2006).

Wedholm *et al.*, (2006) investigated the effect of variations in cow milk protein composition on the cheese yield and clotting properties and found that samples with low concentration of κ-CN showed poor or no coagulation resulting in a weak or no coagulum. Milk with high concentrations of κ-CN, β-CN and αS1-CN results in improvement of cheese-making properties (Wedholm *et al.*, 2006). Buffalo milk shows higher content of κ-CN, which shortens the duration of the enzymatic curding phase and lowers the quantity of rennet needed, compared to cow milk. αS1-CN and β-CN are found in smaller quantities in buffalo milk while κ-CN and αs2-CN are higher in buffalo milk (Zicarelli, 2004). Julien, *et al.*, (1985) reported that the protein composition of casein micelles in cow milk is generally 1κ: 3β: 5α. Heck *et al.*, (2008) measured mean relative protein concentration of milk from 1948 Dutch Holstein-Friesian cows (Table 2). Individual caseins percent of total casein in buffalo and bovine milk were reported by Ståhl-Högberg and Lind (2003) (Table 2).

Table 2 Mean relative protein concentration of buffalo and cow milk

Protein (%)	α -LA	β-LG	αS1-CN	αS2-CN	β-CN	κ-CN
Buffalo milk[1]	ND	ND	30.2	17.6	33.9	15.4
Cow milk[2]	ND	ND	38.4	10.5	36.5	12.5
Cow milk[3]	2.4	8.3	33.6	10.1	27.2	8.4

ND=no data. [1, 2] Ståhl-Högberg and Lind (2003), [3] Heck *et al.*, (2008)

2.6.3 Whey proteins

The major whey proteins in milk are β-lactoglobulin (β-LG) and α-lactalbumin (α-LA). β-LG counts for 80% of total whey proteins and is the most prevalent bovine whey protein. Whey proteins contain also immunoglobulines, which protect the newborn against infections (Ng-Kwai-Hang, 2002). Mawal *et al.*, (1965) reported that whey proteins in buffalo milk are similar in proportions to their counterparts in cow milk. α-LA is a Ca^{2+} binding milk protein, which plays an important role in lactose biosynthesis and regulation of milk secretion. In the lactating mammary gland, α-LA together with galactosyltransferase (GT) form the lactose synthase system, which catalyzes the final step in lactose biosynthesis. α-LA increases the specificity and affinity of GT for glucose. α-LA molecule has a single strong Ca^{2+} binding site and binds other physiologically important cations as Na^{+}, Mg^{2+}, K^{+}, and Mn^{2+} and these cations can compete with the Ca^{2+} binding site (Permyakov and Berliner, 2000). Unlike α-LA, β-LG does not appear to have a definite physiological function and many speculations have been made about its role. β-LG belongs to the lipocalins protein family which has a wide diversity of biological functions mostly ligand-binding functions. It is therefore thought that β-lactoglobulin has a similar function, which can explain the significant quantities of β-LG secreted in milk (Kontopidis *et al.*, 2004).

3. Materials and Methods

3.1 Collection of milk samples

Representative milk samples and bulk milk samples from six Mediterranean buffaloes were delivered from Ängsholm dairy farm located 40 km north of Uppsala, Sweden. Milk samples (evening milking) were collected two times per week (Mondays and Thursdays) into 50 ml Eppendorf tubes. Each tube contained 100 µl of bronopol (2 µl/ml) as a sample preservative. Two 50 ml milk samples were obtained from each buffalo cow at each occasion. Milk samples from each buffalo cow were obtained for not less than a month after calving date (Table 3).

Table 3 Date of calving of each of the six buffaloes and number of samples

Buffalo identification number	14	76	69	74	80	70	Bulk milk
Date of calving	22-Jan	24-Jan	25-Jan	31-Jan	19-Feb	04-Mar	
Number of samples	11	11	10	9	10	6	15

3.2 Preparation of samples

For each milk (individual and bulk) sample at each occasion continuously during the collection period, 0.5 ml milk was pipetted into 1 ml Eppendorf tube for capillary electrophoresis (CE) analysis, 10 g of milk were measured and kept into labeled 10 ml Eppendorf tube for calcium content measurement. Another 0.5 ml milk was pipetted in 1 ml Eppendorf tube for plasmin analysis, 1 ml milk in 1 ml Eppendorf tube for free fatty acids (FFA) measurement. Plasmin and FFA were analyzed in a different study (Blänning and Sandelius, 2015). All these samples were labeled and preserved at - 20 to be used at the time of analysis. Fresh milk was used for analysis of calcium activity and pH.

3.3 Analyses

3.3.1 Milk composition data

Individual and bulk milk samples (50 ml each) were sent to Department of Animal Nutrition and Management (HUV) laboratory every Friday during the collection period to be analyzed for milk composition by MilkoScan FT (FOSSElectric A/S) and for SCC by Fossomatic (Foss FT 120). The analysis method is based on FTIR interferometer (Fourier Transform Infrared Spectroscopy) while counting the SCC is based on the recognition of the DNA of the cells in milk. The milk composition data included in the investigation were gross milk composition; SCC, casein, protein, fat, total solids, lactose and citric acid.

3.3.2 Calcium activity and pH

Measurement of fresh milk pH was done continuously during the collection period by a pH meter (Prolab, 3000 Digital-Multi-Meter, SI Analytics, Germany). Calcium activity measurement (per mV) was based on Ca^{2+} activity in milk-based systems by Ca-ISE as described by (Gao *et al.*, 2011). Calcium activity was measured with calcium ion sensitive electrode (Ca 800 DIN, WTW, Germany). Five standards were used and the electrode was calibrated prior the sample analysis before each measurement occasion as shown in Table 4.

Table 4 Concentrations of $CaCl_2$ and KCl used for calibration of Ca-ISE for milk systems (adapted from Gao *et al.*, 2011)

Calibration solutions	$CaCl_2$ (mM/kg)	KCl (mM/kg)
1	6.6	46.3
2	8.9	43.2
3	10.6	39.6
4	12.2	36.0
5	13.6	32.0

3.3.3 Milk protein analysis

Protein separation was performed with 7100 capillary electrophoresis (CE) system (Agilent Technologies Co. U.S.) as described by Johansson *et al.* (2013). Separations were performed using unfused silica standard capillary, 50 μm inner diameter and 40 cm active length (Chrom Tech, Märsta, Sweden). Result of the protein separation was displayed with Chemstation software version A 10.02 in a CE electropherograms (Agilent Technologies).

3.3.3.1 Preparation of buffer solutions

Urea stock, run buffer and sample buffer were prepared for capillary electrophoresis using the equation (m = M * c * V) where M = the molecular weight, c = buffer concentration and V = the volume. Urea stock was prepared by mixing 126.1 g urea (6 M) with 0.0175 g methylhydroxyethylcellulose, (MHEC, 0.05%) and 6.3 g ion exchange resin (Bio-Rad, California, USA). Purified water was added to the mix to get a urea stock of 0.35 l. The compounds were mixed for 4 h to lower the conductivity by ion exchange resin until the conductivity is less than 2 μS/cm and the mixture was then filtrated. The function of urea is to denature proteins and to modify the electro osmotic flow (EOF) (Landers, 1997). The ion exchange resin decreases ion strength of the buffers and the sample (Bio-Rad Laboratories, 2000). MHEC helps in suppression of electro osmotic flow in the capillary (Landers, 1997).

For the run buffer, 4 g citric acid (0.19 M), 0.59 g trisodium citrate dehydrate (0.02 M), and 0.175 g MHEC (w/w 0.05%) were mixed with 126.1 g urea stock (6.0 M) followed by addition of purified water to obtain 100 ml of run buffer. The function of trisodium citrate dehydrate is to stabilize pH while that of citric acid is to adjust pH (Landers, 1997). For the sample buffer, 4.05 g hydroxymethyl-aminomethane (Tris, 0.167 M), 1.8 g 3-(N-morpholino) propanesulfonic acid (MOPS, 0.042 M), 0.175 g MHEC (0.05%), 5 g ethylene-diamine-tetraacetic acid disodium salt dihydrate (EDTA, 0.067 M), 126.1 g of the urea stock (6.0 M) were mixed altogether.

DL-dithiothreitol (DTT, 0.017 M) was only added to sample buffer before use (0.039g DTT/15ml sample buffer). Purified water was added to obtain 200ml of sample buffer solution. Both sample and run buffer were portioned for use before storage in -20 °C. MOPS and Tris serve as buffering agents to stabilize pH (Landers, 1997). EDTA captures the divalent ions while DTT is a reducing agent that converts dimers of αS2-CN to its monomer forms (Heck *et al.*, 2008).

3.3.3.2 Preparation of the samples

Milk samples were defrosted overnight in 4 °C then kept in a water bath for 15 minutes at 45 °C. Each sample was convulsed with a vortex mixer (Vortex-Genie 2, Scientific Industries, Inc., U.S.) and was warmed in water bath for another 15 minutes at 45 °C. After the water bath, 150µl of each milk sample was pipetted into respective labeled Eppendorf safe lock tubes (Eppendorf, Germany) followed by the addition of 350 µl of sample buffer and adding 0.039 g/15ml DTT. Each sample was once again convulsed with the vortex mixer and incubated for 1h in room temperature. After incubation step, the samples were defatted by centrifugation (Himac CT15RE, Hitachi Koki Co., Ltd.) at 10 000 rpm for 10 minutes at 4 °C. The creamy layer on top was then removed by cotton swabs. Each sample was filtered by using a syringe with a 45µm nylon membrane filter. Thereafter, 30µl was pipetted into labeled conical vials (Agilent, Kista Sweden) for protein analysis by the capillary electrophoresis instrument.

3.3.4 Analysis of calcium content

Milk samples were analyzed for calcium content by atomic absorption spectrometric method based on IDF (International Dairy Federation) 119: 2007 and IDF 154: 1992. Each frozen milk sample (10 g) was freeze dried to obtain a fine powder. The freeze drier (Labconco, Ab Nino Lab) was set to 0 °C in which the samples were kept under vacuum for three days.

3.3.4.1 Ashing step

Heat resistant crucibles were used to ash the dry milk samples. The crucibles were washed with distilled water and heated up at 500 °C in the oven (Nabertherm controller B 180) with a layer (2 ml) of 10% nitric acid (NA) to remove calcium ions and dirt. After heating the crucibles, they were washed with distilled water and let to dry prior use. Afterwards, the 10 g freeze dried milk of each sample were put into a corresponding crucible and heated in the oven at 550 °C for 90 minutes until a white ash was received.

3.3.4.2 Preparation of sample and standard solutions

A stock solution of calcium carbonate ($CaCO_3$) 1 g/l was made by diluting 0,25 g of $CaCO_3$ to 100 ml distilled water and kept in special brown bottles to be protected from light breakdown of the stock. A standard solution was used for the calibration curve consisted of 10 ml of stock solution mixed with 5 ml of 25% NA solution and diluted to 100 ml with distilled water and kept in special brown bottle. Six calibration solutions were made (0, 1, 2, 3, 4, 5) ml of the previous solution were pipetted into six separate brown bottles respectively and all diluted to 100 ml with distilled water. Thus the concentrations of the standard solutions were 0 µg, 1 µg, 2 µg, 3 µg, 4 µg and 5 µg per liter. These standard solutions were used for calibration prior each measurement occasion. All the glassware and plastics used were kept in 10% NA solution overnight before measurement. All of them were rinsed three times with distilled water and left to dry before use. After the ashing step, the obtained ash was dissolved in 1 ml

of 25% NA solution and the crucible content was transferred into a labeled 250 ml volumetric flask by properly rinsing the crucible with distilled water for three times. Distilled water was further poured into the flasks to dilute up to the 250 ml mark followed by rough mixing.

Five ml of the sample solution was into a 100 ml volumetric flask together with 10 ml of lanthlanthanum chloride (lanthanumIIIchloride heptahydrate, 27 g/L, Sigma-Aldrich) and diluted to the 100 ml mark with distilled water. The total calcium content was analyzed in an atomic absorption spectrophotometer (AAS) (Perkin Elmer, A-Analyst 100) with a wavelength of 422.7 nm, provided with a calcium lamp.

3.4 Statistical evaluation

Basic statistical analyses were performed. Mean and standard deviations of different parameters (milk composition data, pH, total calcium content, ionic calcium concentration, protein) for all the milk samples (individual and bulk) were presented using Microsoft Excel.

4. Results

4.1 Milk composition data

The total fat content ranged between 6.76% and 7.38% (Table 5) with an average of 6.97% in milk from individuals while the bulk milk fat content was 6.61%. No particular trend was observed for the fat content throughout the sampling period. Protein content of the bulk milk was on average 4.31% and for the individual buffalo cows varied between 4.26% and 5.75% (Table 5). The protein and casein contents showed a decrease along the sampling period by 33.8% and 7.8% respectively (Figure 2). Lactose content varied between 4.24% and 5.15% for the individual buffalo cows while the average bulk milk was 5.08%. Lactose increased throughout the measurement period by 14.6%, scoring an average of 4.40% at the start of lactation and then increased to 5.16% at the later period (Figure 2). SCC could only be measured during the first half of the collection period due to technical problems with the instrument, yet the SCC was high for all buffalo cows at the very beginning of the sampling period and decreased continuously. This was true for all the individuals except for buffalo number 14, which showed much elevated counts than the others throughout the first half of the collection period. Despite the high SCC in milk from buffalo number 14, the bulk milk was not greatly affected probably due to dilution by other individuals' milk of lower count (Table 5). Citric acid of the bulk milk was on average 0.18% and for the individual buffalo cows varied between 0.14% and 0.19%. TS of the bulk milk was on average 16.64% and for the individual buffalo cows varied between 16.62% and 17.61% (Table 5).

Table 5. Measured average concentrations of total fat, total protein, total casein, lactose, citric acid, somatic cell count and total solids in bulk milk and milk from individual buffalo cows, mean and standard deviation

Buffalo	Total fat (%)	Total protein (%)	Total casein (%)	Lactose (%)	Citric Acid (%)	SCC	TS (%)
Bulk	6.61±1.07	4.31±0.30	3.74±0.14	5.08±0.20	0.18±0.01	101.6±218.57	16.64±1.0
70	6.82±1.44	5.75±0.36	4.48±0.22	4.24±0.24	0.15±0.02	106.42±84.94	17.45±1.23
80	6.86±0.54	4.26±0.50	3.73±0.32	5.15±0.26	0.19±0.03	12.5±4.84	16.80±0.63
14	6.76±0.26	4.68±0.44	3.68±0.11	4.79±0.24	0.19±0.02	440±602.48	16.92±0.49
69	6.77±0.49	4.26±0.38	3.69±022	5.01±0.19	0.19±0.01	44.4±9.01	16.62±0.65
74	7.25±0.26	4.89±0.33	4.17±0.13	4.94±0.29	0.14±0.01	71.8±29.10	17.61±0.29
76	7.38±0.52	4.47±0.38	3.90±0.21	4.93±0.24	0.16±0.01	106.66±140.78	17.24±0.62
Average*	**6.97±0.43**	**4.72±0.06**	**4.44±0.07**	**4.84±0.03**	**0.17±0.006**	**130.29±229.99**	**17.11±0.31**

* Average per all buffalo cows.

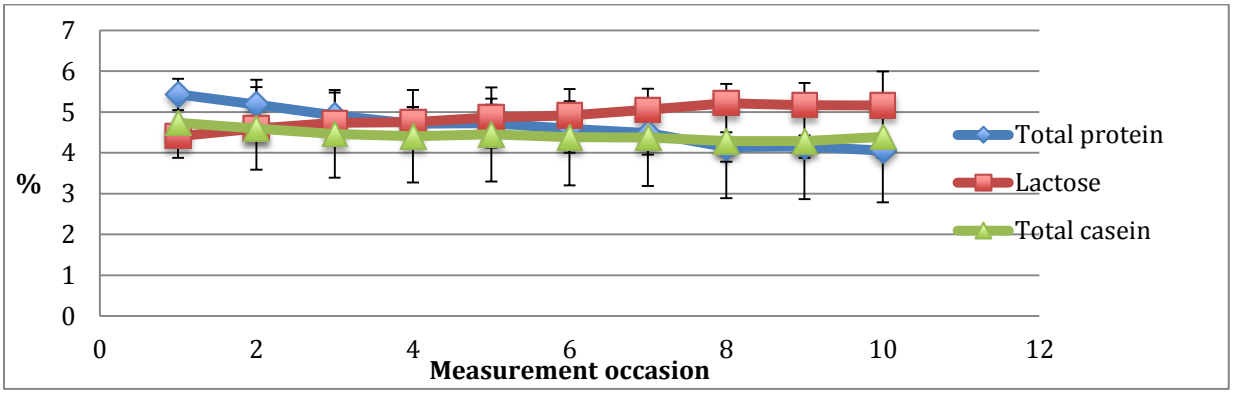

Figure 2 Change in average total protein, lactose and total casein in buffalo milk during the first five weeks after calving. Error bars denote standard deviation.

4.2 Total calcium and pH

As shown in Table 6, the pH of the bulk milk was 6.80 in average and ranged from 6.58 to 6.78 in the individual buffalo cows with average pH of 6.70. The average pH increased throughout the collection period by 5.7% as the average pH of the first collected milk samples (for all the buffalo cows) was 6.43. In the middle of samples collection period, the pH increased to 6.72 while a pH of 6.82 was reached at the end of collection period (Figure 3). The total calcium content in buffalo bulk milk was 1.57±0.036 g kg^{-1} in average. The total calcium content measured in individual milk varied between 1.52-1.75 g kg^{-1} and the mean concentration was 1.63 g kg^{-1} (Table 6). The total calcium content showed a 5% decline during the sampling period as the average total calcium content of the first collected milk samples was 1.66 g kg^{-1} whereas at the end of the collection period, it reached 1.58 g kg^{-1} (Figure 3).

Table 6 Measured average concentrations of pH, total calcium content and calcium activity in bulk milk and milk from individual buffalo cows, mean and standard deviation

Buffalo ID	pH	Total Calcium (g kg^{-1})	Calcium Activity (mM)
Bulk	6.80±0.093	1.57±0.063	2.10±0.978
70	6.58±0.181	1.75±0.143	2.99±0.988
80	6.78±0.131	1.73±0.100	3.06±0.473
14	6.68±0.117	1.52±0.171	2.62±0.326
69	6.72±0.130	1.66±0.100	2.60±0.209
74	6.75±0.139	1.59±0.145	2.66±0.275
76	6.68±0.166	1.55±0.108	2.66±0.239
Average*	**6.70±0.024**	**1.63±0.029**	**2.76±0.294**

* Average per all buffalo cows.

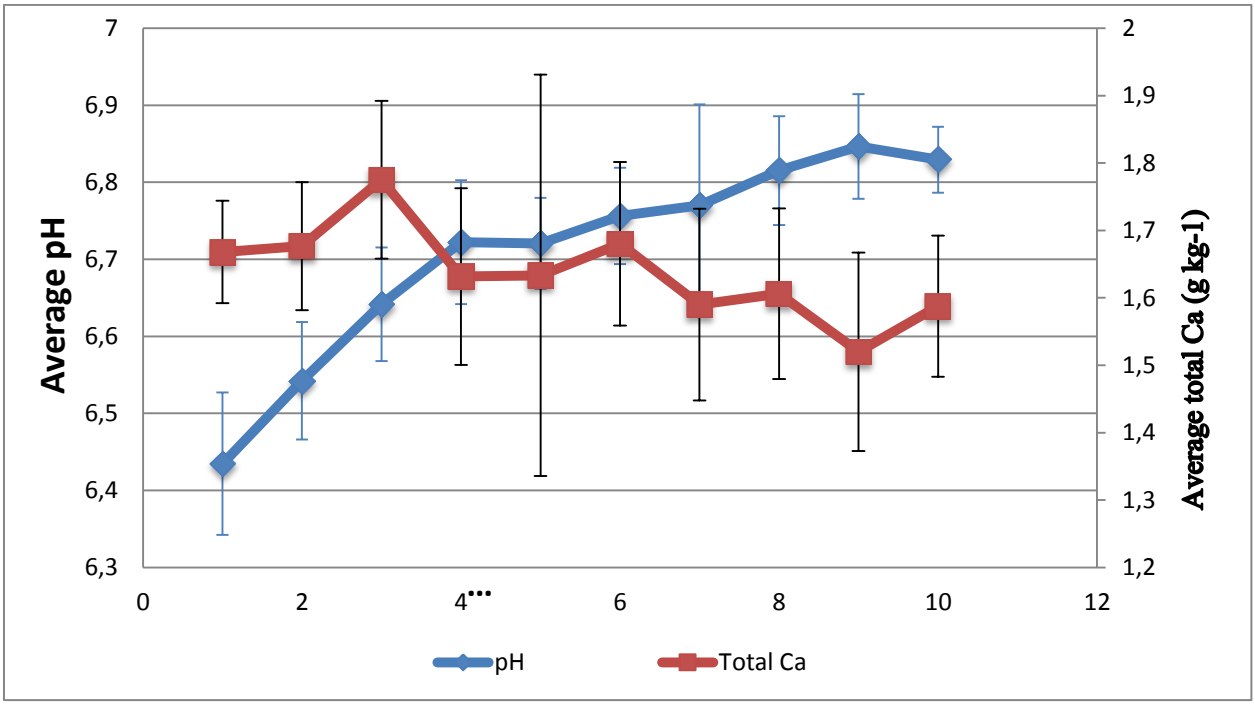

Figure 3 Change in average pH and average total calcium content in buffalo milk during the first five weeks after calving. Error bars denote standard deviation.

4.3 Calcium activity

Calcium activity in bulk milk was 2.10±0.978 mM (0.084 g kg^{-1}) and varied between 2.60 mM to 3,059 mM and the mean calcium activity was 2.76 mM (Table 6). Calcium activity has declined by 25.4% during the collection period (Figure 4).

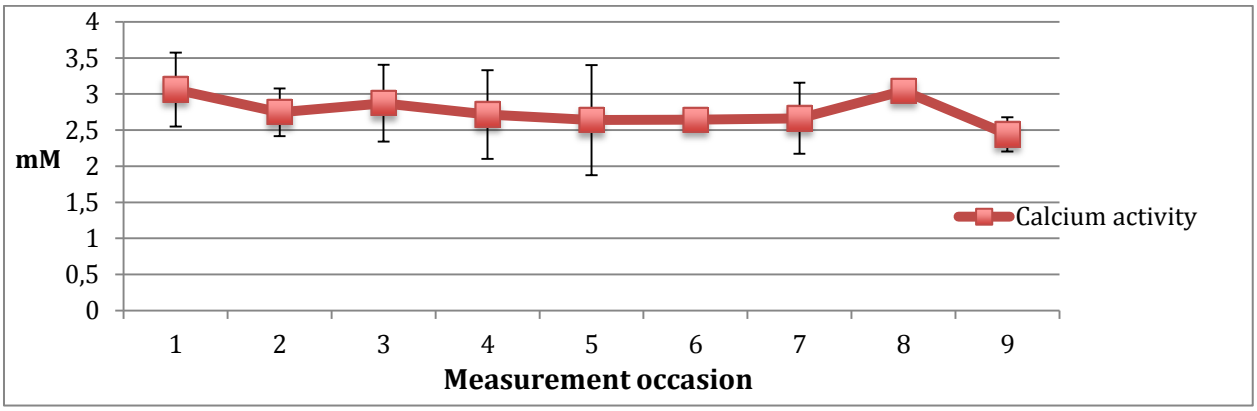

Figure 4 Change in average calcium activity in buffalo milk during the first five weeks after calving. Error bars denote standard deviation.

4.4 Milk protein analysis

Proteins were separated with retention times between 11 and 25 minutes, shown in Figure 5.

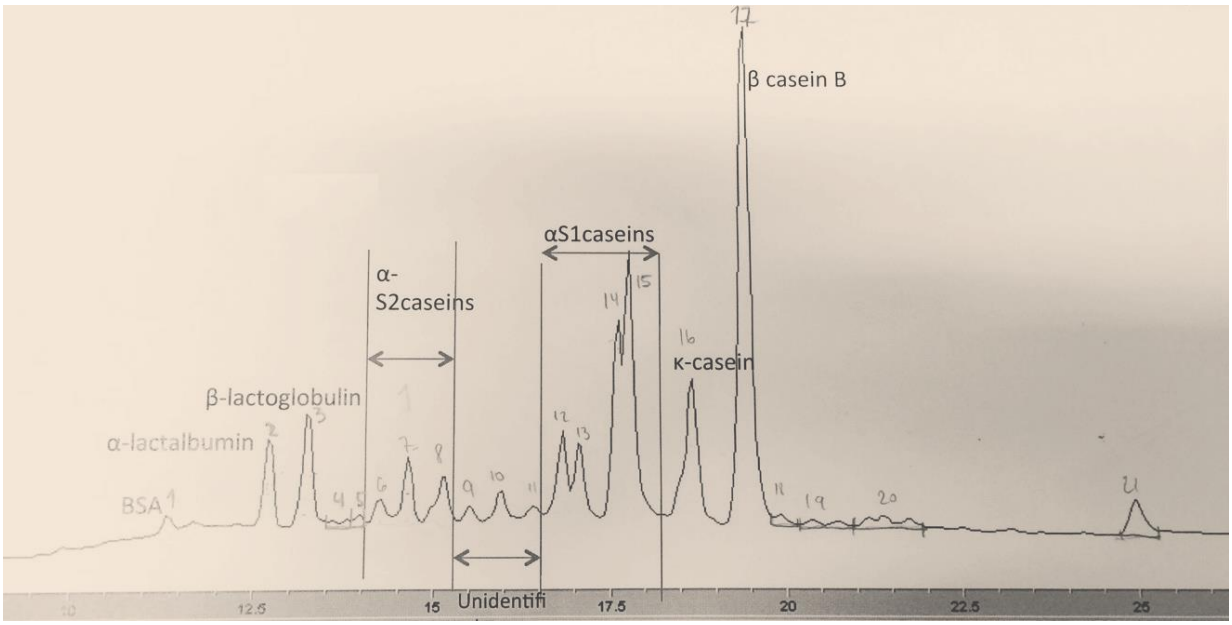

Figure 5 Representative capillary electrophoresis electropherogram for buffalo milk with UV absorption peaks addressed to milk proteins. Identified caseins and whey proteins are indicated in the figure: αS1- CN, αS2- CN, β-CN, κ-CN, α-LA and β-LG.

4.4.1 Relative milk protein concentrations

The mean relative concentrations of caseins and whey proteins (total and individual proteins) changed to a various extent between the individual buffalo cows, which reflects the individual variation between them.

4.4.1.1 Individual caseins and whey proteins relative concentrations

The mean relative concentrations of caseins were: 36.6% for αS1-CN, 28.7% for β-CN, 8.7% for αS2-CN and 8.2% for κ-CN (Figure 6). The ratio between κ-CN: β-CN: αS-CN was 1: 3.5: 5.5.

The mean relative concentrations of whey proteins were: 4.4% for α-LA and 8.4% for β-LG (Figure 6). The mean relative concentrations (%) of individual and bulk buffalo milk caseins and whey proteins analyzed during the first five weeks after calving are shown in Figures 7, 8, 9, 10, 11 and 12. Results show individual variation in the relative concentration of the main milk proteins. Average relative concentration per all buffalo cows is also shown for each protein.

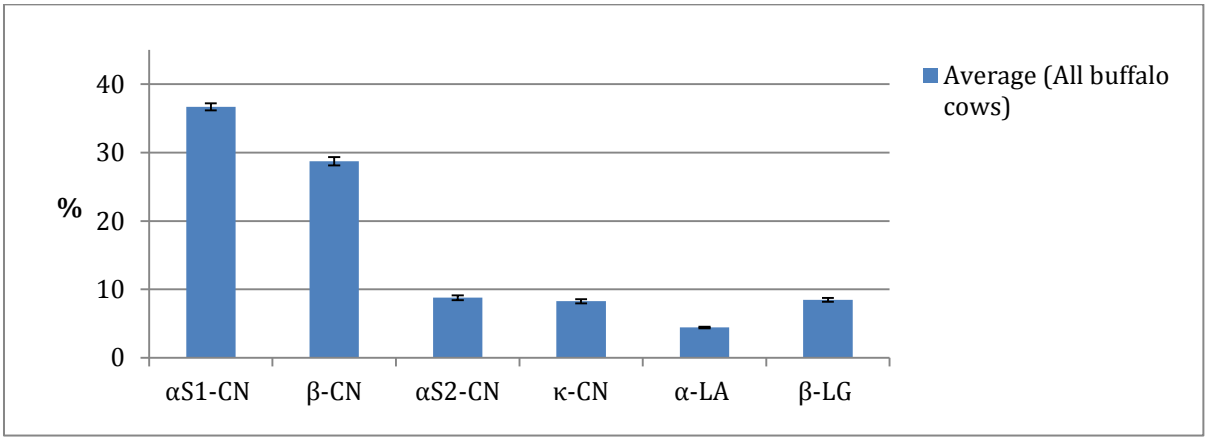

Figure 6 Mean relative concentrations of individual buffalo milk caseins and whey proteins analyzed during the first five weeks after calving. Error bars denote standard deviation.

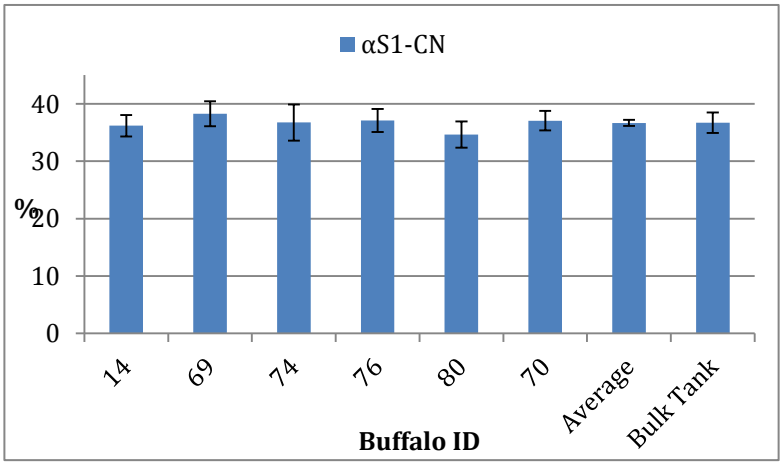

Figure 7 Mean αS1-CN relative concentration in individual and bulk buffalo milk. Error bars denote standard deviation.

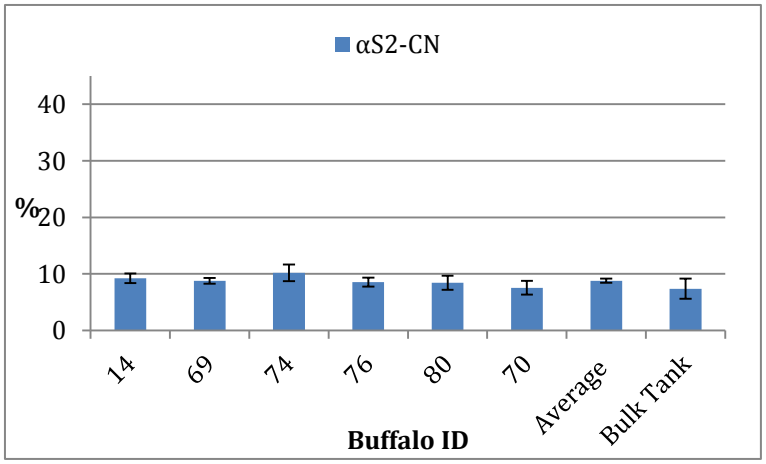

Figure 8 Mean αS2-CN relative concentration in individual and bulk buffalo milk. Error bars denote standard deviation.

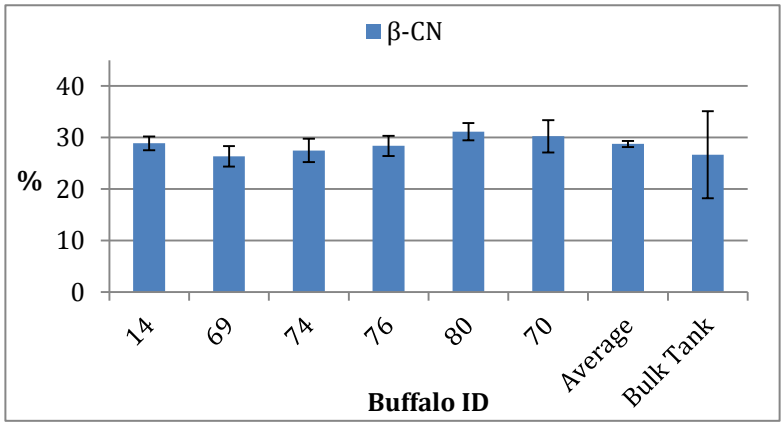

Figure 9 Mean β-CN relative concentration in individual and bulk buffalo milk. Error bars denote standard deviation.

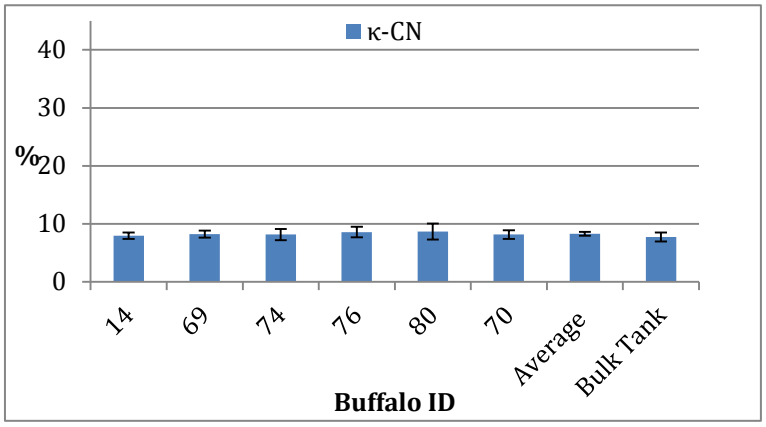

Figure 10 Mean κ-CN relative concentration in individual and bulk buffalo milk. Error bars denote standard deviation.

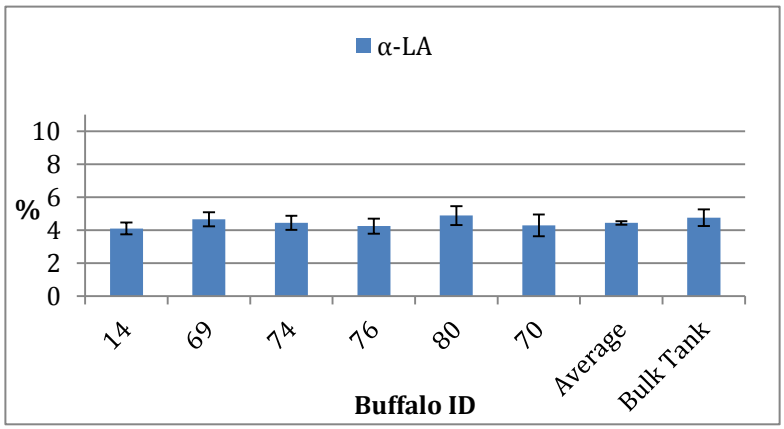

Figure 11 Mean α-LA relative concentration in individual and bulk milk. Error bars denote standard deviation.

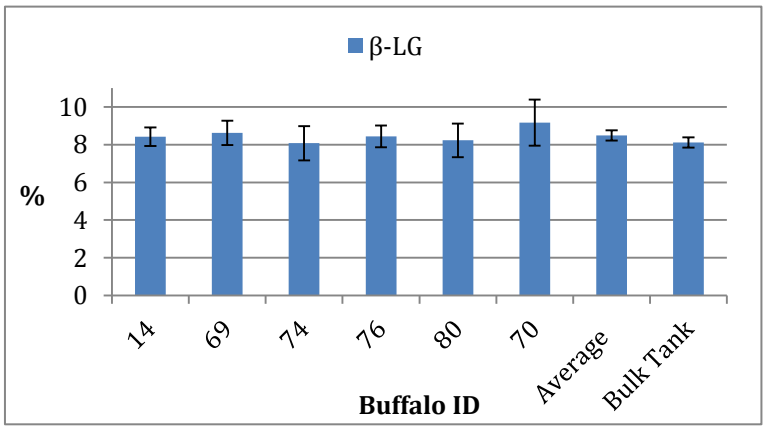

Figure 12 Mean β-LG relative concentration in individual and bulk buffalo milk. Error bars denote standard deviation.

4.4.1.2 Total casein and total whey protein relative concentrations per individual animals

The relative mean concentration of total caseins in the individual buffaloes (Figure 13) ranged between 81.6% (buffalo 69) and 82.99% (buffalo 70). The mean relative concentration of total caseins in the bulk milk was 78.4%. The relative mean concentration of total whey proteins in the individual buffaloes ranged between 12.51 (buffalo 14) and 13.46% (buffalo 70) while the mean relative concentration of total whey proteins in the bulk milk was 12.8% (Figure 14).

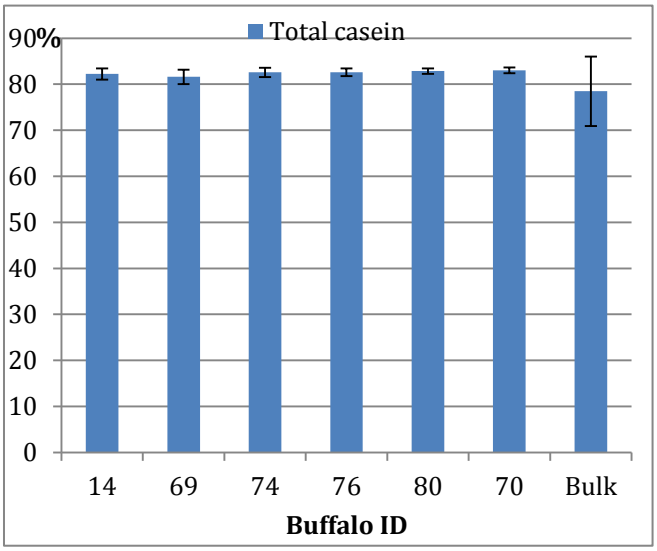

Figure 13 Average total casein relative concentrations calculated per animal. Error bars denote standard deviation.

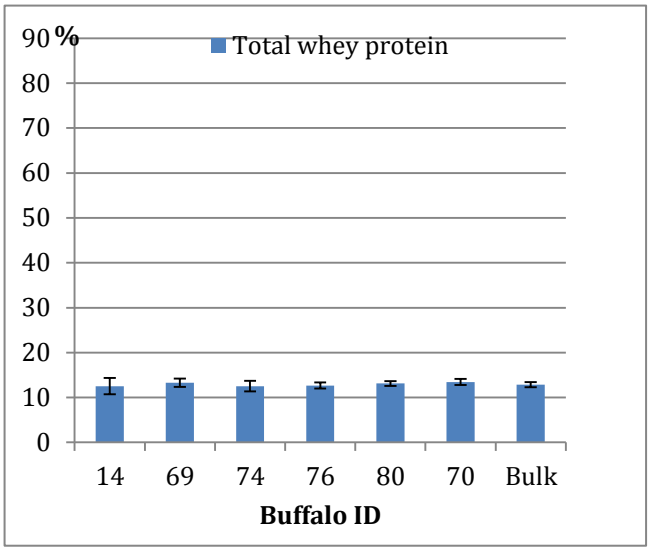

Figure 14 Average total whey proteins relative concentrations calculated per animal. Error bars denote standard deviation.

4.4.2 Changes in caseins and whey proteins during sampling period

There were changes in the relative total and individual caseins and whey proteins relative concentrations from the start to the end of the sampling period.

4.4.2.1 Changes in total casein and total whey protein

Total casein declined during the sampling period by 2.2%. The total relative casein concentration scored 82.7% at the beginning of collection and decreased to 80.9% (Figure 15). Unlike total casein, the total relative whey protein concentration showed an increase (4.7%) during the collection period. Total whey proteins started with 12.5% and increased to 13.2% at the end of the collection period (Figure 16).

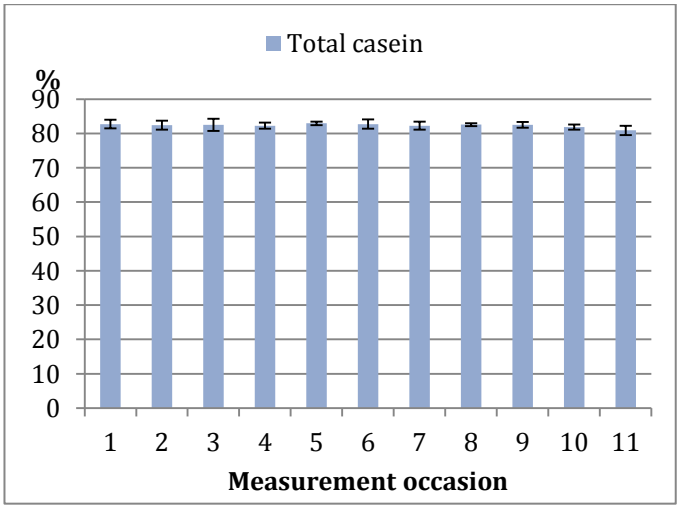

Figure 15 Changes in relative total casein concentration calculated per day of sampling. Error bars denote standard deviation.

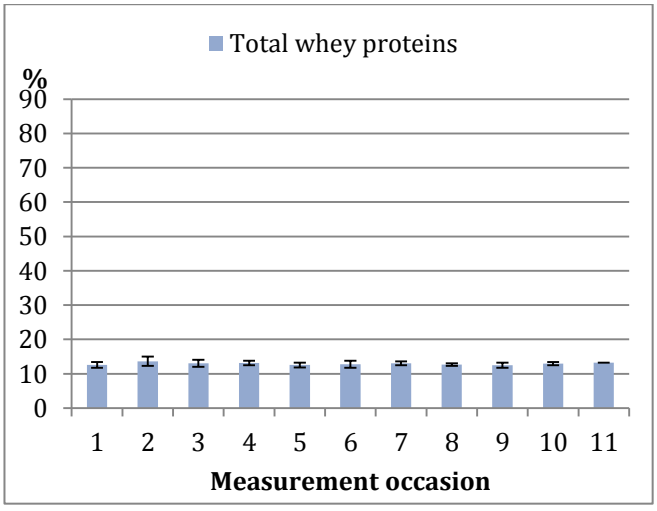

Figure 16 Changes in relative total whey protein concentration calculated per day of sampling. Error bars denote standard deviation.

4.4.2.2 Changes in the individual caseins and whey proteins

Both αS1-CN and αS2-CN showed a decrease in the relative concentration over time (Figure 17, 18). αS1-CN declined by 8.7% while αS2-CN declined by 3.4% during the sampling period. β-CN showed an increase by 5.4% during the sampling period (Figure 19). κ-CN increased by 8% from the start of sampling to the fourth week of sampling but declined slightly at the end of the sampling period (Figure 20). α-LA showed an increase (18.7%) (Figure 21) in contrast to β-LG, which showed 3.1% decrease during the collection period (Figure 22).

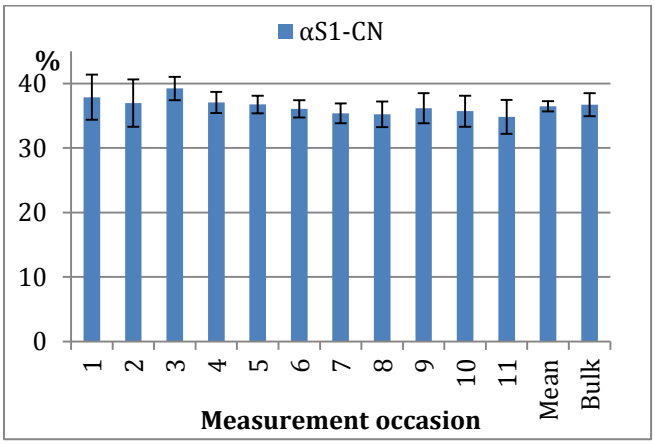

Figure 17 Changes in αS1-CN relative concentration per day of sampling. Error bars denote standard deviation.

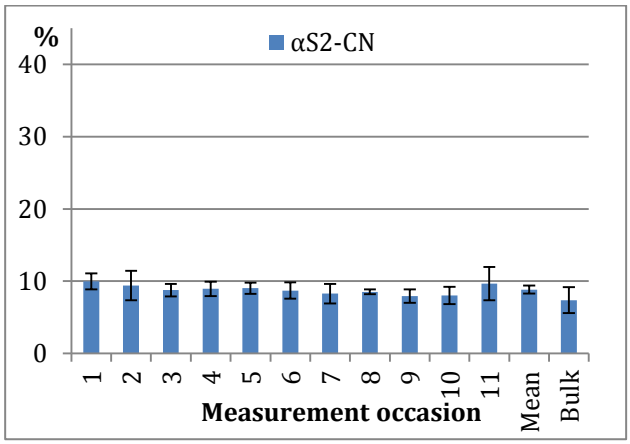

Figure 18 Changes in αS2-CN relative concentration per day of sampling. Error bars denote standard deviation.

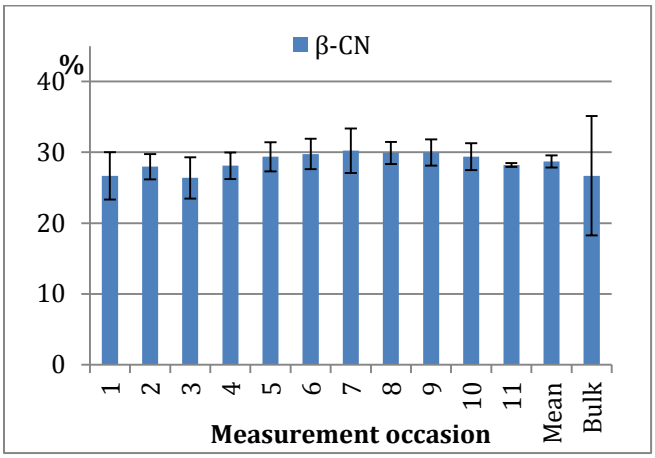

Figure 19 Changes in β-CN relative concentration per day of sampling. Error bars denote standard deviation.

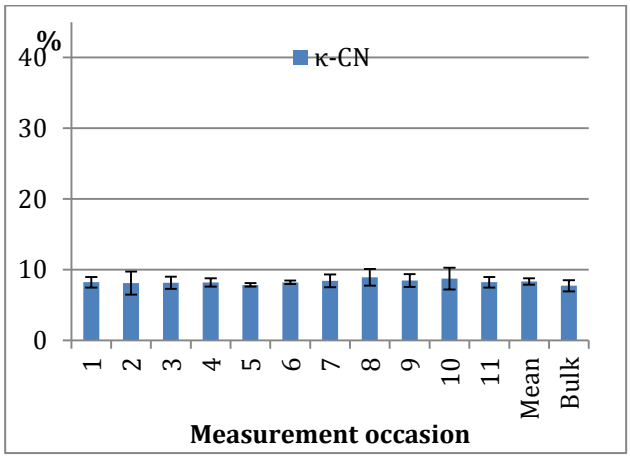

Figure 20 Changes in κ-CN relative concentration per day of sampling. Error bars denote standard deviation.

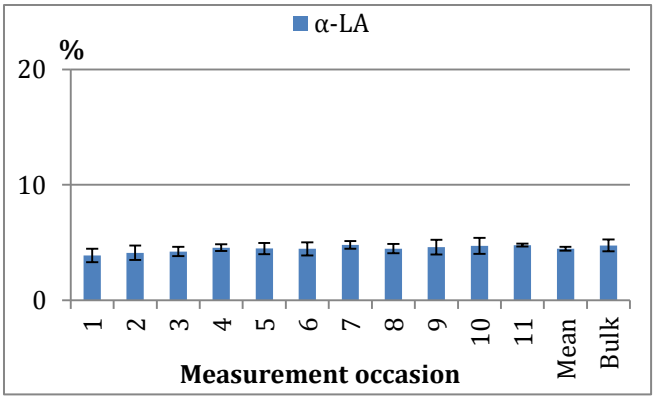

Figure 21 Changes in α-LA relative concentration per day of sampling. Error bars denote standard deviation.

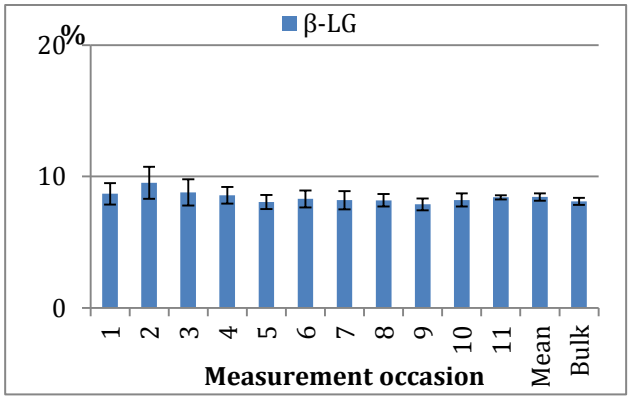

Figure 22 Changes in β-LG relative concentration per day of sampling. Error bars denote standard deviation.

5. Discussion

5.1 Milk composition data

The fat content of the buffalo milk (6.61%) was lower than reported by Thomas (2008) (7-8%) and Varrichio *et al.*, (2007) (8.3%). The protein content (4.31%) was within the range 4.2 to 4.5% reported by Thomas (2008). The casein content was 3.74% and the casein index (casein content/protein content x 100) was 86.7%, which agree with Thomas (2008) who indicated that the casein index of buffalo milk is higher than 80%. The lactose content (5.08%) and the TS (16.6%) were similar to Thomas (2008) (5%) and (16%) respectively. Changes in the fat content (no specific trend), total protein (decline) and lactose content (increase) (Figure 2) during the sampling period were consistent with the findings of Tsioulpas *et al.*, (2007a) in cow milk during 30 days postpartum. The bulk milk value was different from the average values of different parameters measured in individuals. This could be explained by the fact that bulk milk is the summary of milk from different individuals.

5.2 Total calcium and pH

The pH (6.80) is consistent with the pH of buffalo milk reported by Ahmad *et al.*, (2008) (6.81). There was an increase of the pH throughout the collection period by 5.7% meanwhile the total calcium content showed a 5% decrease during the sampling period (Figure 3). The steady increase in pH and decline in total calcium content during the sampling period were consistent with the findings of Tsioulpas *et al.*, (2007a) in cow milk during 30 days postpartum. The inverse relationship between pH and total calcium (Figure 3) has previously been described by Ahmad *et al.*, (2008) and Fox and McSweeney (1998). According to literature, obtaining optimal pH before renneting is crucial to get a gel of suitable consistency. The clotting is fast and constant up to pH 6.55 whereas the clotting time is prolonged as pH increases. At pH values above 6.80, the milk ability to coagulate by rennet is little or even lost (Tsioulpas *et al.*, 2007b). The pH value of milk in the current study was 6.80, which therefore requires careful and accurate reduction during preacidification to the optimal pH before rennet

addition. This is to avoid starting the coagulation process with a high pH resulting in low calcium content in the whey rendering the curd with stretchability problems.

The total calcium content of buffalo milk was 1.57 g kg^{-1} (Table 6), which is 32% lower than reported by Elvingson (2014) (2.3 g kg^{-1}) during late lactation. It was also lower than the values previously reported by Spanghero and Susmel (1996) (2.03 g kg^{-1}) and Ariota *et al.,* (2007) (1.7 g kg^{-1}) of Italian buffalo milk in mid lactation. The total calcium content of buffalo milk in the current study was 23% higher than values recorded by Tsioulpas *et al.,* (2007a) for cow milk (1.21 g kg^{-1}) on day 30 after calving.

Stretchability of the curd depends on curd pH, which depends on calcium content. The curd will not stretch in hot water until demineralization of calcium and sufficient calcium phosphate has ben solubilized from the curd. Despite that the total calcium content of the present study (1.57 g kg^{-1}) was lower than values reported by other workers in mid an late lactation, it was much higher than the average calcium concentration of noncoagulating cow milk samples (1.00 g kg^{-1}) reported by Wedholm *et al.,* (2006).

As mentioned earlier, the initial pH (6.8) of the milk requires preacidification before renneting. Calcium demineralization out of the curd increases with the decrease in pH until obtaining the suitable pH before renneting. After rennet addition, pH continues to decrease meanwhile calcium declines in the curd and drained out to the whey. Starting acidification with total calcium content of 1.57 g kg^{-1} and a pH of 6.8 could possibly lead to decrease in calcium content in the curd to a value close to that reported by Wedholm *et al.*, (2006) (1.00 g kg^{-1}) to reach a pH of 5.0-5.2. This could be investigated in the future through measuring calcium content in the milk and in the whey during the first month after calving.

Milk with impaired clotting properties and noncoagulating milk has been reported in many studies especially in milk from cows in late lactation (Okigbo *et al.,* 1985; Wedholm *et al.,* 2006; Tsioulpas *et al.,* 2007b; Lucey and Fox, 1993). According to Okigbo *et al.,* (1985), reducing the pH of cow milk to 6.3 before coagulation lead to a remarkable decline in coagulation time but it did not increase the curd firmness. Nongelation exhibited by milk after the pH reduction could be explained by either extensive proteolytic activity of indigenous enzymes in milk (resulting in peptones and gamma-caseins as in late lactation cow milk), or due to inappropriate salt balance in milk resulting in poor casein micelles aggregation. Buffalo milk of the current study was analyzed for the concentration of plasmin (PL) and plasminogen (PG) in a separate study. The analysis revealed that no increase of the enzymatic activity could be seen in the first period of lactation (Blänning and Sandelius, 2015). Thus, the enzymatic activity should not affect the mozzarella manufacturing of milk from early lactation.

Some modifications have been suggested by some workers to overcome the poor milk clotting. Lucey and Fox (1993) experimented the pH adjustment and addition of $CaCl_2$ in order to restore the gel firmness of late lactation poor coagulating cow milk but these modifications did not succeed to restore the curd firmness. This result was consistent with results reported by Okigbo *et al.,* (1985) that neither reduction of the pH to 6.3 before coagulation nor addition of $CaCl_2$ led to increase in curd firmness. Mixing of milk with poor coagulation with an equal amount of milk with good coagulation could not succeed either as it resulted in noncoagulating milk. Okigbo *et al.,* (1985) indicated that 86.9% increase in gel firmness was gained by application of the following modifications altogether: pH reduction, addition of $CaCl_2$, and reducing chymosin concentration. Reducing of chymosin could be

explained by proteolysis of the caseins and thus poor coagulation when adding high concentration of rennet to the milk with a reduced pH. Whether this solution could be beneficial to overcome the poor stretchability of buffalo milk in the first lactation needs further investigation.

5.3 Calcium activity

Calcium activity in bulk milk was 2.10±0.978 mM (Table 6), which was higher than the average calcium activity recorded for bovine milk; 1.26 mM reported by Tanaka *et al.*, (2011) and 2.0 mM by Holt *et al.*, (1981). The ratio between calcium activity to total calcium content (1.57 g kg^{-1}) was 5.35%, which was 8% higher than reported by Tanaka *et al.*, (2011) (4.92%) for cow milk and 22% lower than the value recorded for cow milk by Holt *et al.*, (1981) (6.9%). Calcium activity has declined by 25.4% (Figure 4) whereas lactose content increased throughout the measurement period by 14.6% (Figure 2). Thus, a negative correlation between calcium activity and milk lactose was observed, which is consistent with results reported by Tanaka *et al.*, (2011). The total calcium content showed a 5% decline during the sampling period meanwhile calcium activity has declined by 25.4% indicating a positive correlation between them. Tanaka *et al.*, (2011) found no correlation between total calcium content and calcium activity.

Tsioulpas *et al.*, (2007b) described the relationship between calcium activity and pH in milk and revealed that the calcium activity decreases with the pH increase. Findings of the present study were consistent because the pH increased as calcium activity declined during the sampling period. Tsioulpas *et al.*, (2007b) reported also that the curd becomes harder with the lower the pH or the higher the Ca^{2+} concentration in milk. It was also found that if the Ca^{2+} level is lower than 1.5 mM, no coagulation will occur. Accordingly, calcium activity of milk in the present study (2.10±0.978 mM) would be in favor of a harder gel.

5.4 Milk protein analysis

In this study, κ-CN mean relative concentration (8.2%) (Figure 6) in buffalo milk was 46% lower than reported by Ståhl-Högberg and Lind (2003) (15.4%). It was also 34% lower than cow milk value (12.5%) reported by the same authors, whereas it was very close to the value (8.4%) obtained by Heck *et al.*, (2008) for cow milk. Just as κ-CN, β-CN mean relative concentration (28.7%) (Figure 6) was 15% lower than that of buffalo (33.9%) and than cow (36.5%) milks reported by Ståhl-Högberg and Lind (2003) while it was slightly higher than (27.2%) reported by Heck *et al.*, (2008) for cow milk.

αS1-CN was 36.6%, which is higher than that in buffalo milk (30.2%) and lower than (38.4) in cow milk reported by Ståhl-Högberg and Lind (2003) and higher than cow milk (33.6%) recorded by Heck *et al.*, (2008). αS2-CN (8.7%) was much lower than that (17.6%) of buffalo milk reported by Ståhl-Högberg and Lind, (2003). αS2-CN (8.7%) was much lower than that in buffalo milk reported by Ståhl-Högberg and Lind (2003) (17.6%). It was also lower than αS2-CN concentrations in cow milk (10.5% and 10.1%) reported by Ståhl-Högberg and Lind (2003) and Heck *et al.*, (2008) respectively. Regarding the whey proteins, α-LA (4.4%) was higher than cow milk (2.4%) recorded by Heck *et al.*, (2008), while β-LG (8.4%) was almost the same concentration.

According to Julien *et al.*, (1985), the protein composition of casein micelles in cow milk is generally 1κ: 3β: 5α-CN and the protein composition of buffalo milk in this study was slightly close to this composition (1κ: 3.5β: 5.5α-CN) and also close to the protein composition of cow milk reported by Heck *et al.*, (2008) (1κ: 3.2β: 5.2α-CN). In contrast, the protein composition of buffalo milk reported by Ståhl-Högberg and Lind (2003) had a markedly different composition of 1κ: 2.2β: 3.1α-CN.

The results of protein analysis in this study shows that buffalo milk in the first five weeks after calving is considerably different concerning protein composition than in buffalo milk according to literature (Ståhl-Högberg and Lind, 2003; Zicarelli, 2004). Thus, milk with such a composition would affect cheese making properties negatively, being lower in κ-CN and β-CN. This finding could be supported by findings by Wedholm *et al.*, (2006) who found that samples with low concentration of κ-CN showed poor or no coagulation resulting in a weak or no coagulum. Accordingly, buffalo milk during the first five weeks after calving might negatively affect the cheese-making properties rendering the milk with increased rennet clotting time and poor coagulation if any.

6. Conclusion

From the findings of this study it was revealed that the total calcium content of buffalo milk during the first five weeks of lactation was lower than reported for the average buffalo milk. Milk protein analysis showed that buffalo milk in the first period of lactation is considerably different concerning concentration of caseins than the reported concentration in buffalo milk being lower in κ-CN and β-CN concentrations. The result of calcium activity of the milk was found to be in favor of obtaining a harder gel. The lower total calcium content in combination with the difference in protein concentration (low κ-CN and β-CN concentrations) could explain the reason behind the poor coagulation properties of buffalo milk during the first period of lactation in relation to mozzarella cheese manufacturing

7. Future research

A bigger study involving a larger number of animals and extending the sampling period to mid and late lactation is needed. This would help to get a better understanding on how the buffalo milk composition (especially calcium content in milk, calcium content in whey, calcium activity, pH and protein profile) differs from lactation period to another. A longer sampling period with more samples and more buffaloes would be beneficial to investigate the development of milk quality and suitability for mozzarella production over lactation. Reducing concentration of the rennet added to milk together with pH reduction and addition of $CaCl_2$ to enhance the poor clotting properties of milk need further investigation.

8- Acknowledgements

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